Chapter 4
Signaling Cascades: Consequences of Varying Substrate and Phosphatase Levels

Elisenda Feliu, Michael Knudsen, and Carsten Wiuf

Abstract We study signaling cascades with an arbitrary number of layers of one-site phosphorylation cycles. Such cascades are abundant in nature and integrated parts of many pathways. Based on the Michaelis–Menten model of enzyme kinetics and the law of mass-action, we derive explicit analytic expressions for how the steady state concentrations and the total amounts of substrates, kinase, and phosphatates depend on each other. In particular, we use these to study how the responses (the activated substrates) vary as a function of the available amounts of substrates, kinase, and phosphatases. Our results provide insight into how the cascade response is affected by crosstalk and external regulation.

1 Introduction

Reverse phosphorylation of proteins is one of the principal mechanisms by which signals are transmitted in living cells. Signaling pathways typically contain a cascade of phosphorylation cycles involving kinases and phosphatases, where the activated (in general, the phosphorylated) protein in one layer acts as the kinase in the next layer. The levels of substrate, phosphatase or stimulus in these cycles might be regulated externally by other proteins. Many disease-related proteins are

E. Feliu (✉)
Bioinformatics Research Centre, Aarhus University, Aarhus, Denmark
e-mail: efeliu@birc.au.dk

M. Knudsen • C. Wiuf
Bioinformatics Research Centre, Aarhus University, Aarhus, Denmark

Centre for Membrane Pumps in Cells and Disease (PUMPKIN), Aarhus University, Aarhus, Denmark
e-mail: michaelk@birc.au.dk; wiuf@birc.au.dk

I.I. Goryanin and A.B. Goryachev (eds.), *Advances in Systems Biology*,
Advances in Experimental Medicine and Biology 736,
DOI 10.1007/978-1-4419-7210-1_4, © Springer Science+Business Media, LLC 2012

part of signaling pathways, for example, the tumor suppressor proteins BRCA1 and p53 exist in many phosphoforms and the PTEN protein is a phosphatase. Cascade malfunctioning might therefore be a cause of disease (e.g., [1,2]). It is a goal of this work to understand how a signaling cascade adjusts to changes in the amount of initial stimulus or the amount of phosphatase and substrate in specific layers.

The biological relevance of this signaling mechanism is well-established theoretically [3–7]. Properties of general signaling cascades, such as ultrasensitivity and signal amplification, might be elucidated from the study of signaling cascades with an arbitrary number of layers n, where each layer is a one-site phosphorylation cycle. Such cascades are part of many pathways [8, page 342], [9, 10] and have been investigated mathematically: $n = 1$, e.g., [11,12], $n = 1, 2$, e.g., [5,13,14], and arbitrary n, e.g., [3, 7, 15, 16]. In much previous work, a cascade is modeled as a system of independent layers, thereby ignoring the effect of kinase sequestration. This was pointed out in [7]. This simplification further implies that one cannot study how activation of one layer effects the concentration levels in the layers of the upstream. To study this it is crucial to consider connected layers.

Here, we give an analysis of a cascade with n connected layers. We provide analytic expressions for how species concentrations and total amounts of substrates and phosphatases are related. Specifically, we use Michaelis–Menten's classical model of an enzyme reaction which includes the formation of intermediate complexes (thus accounting for sequestration). Based on mass-action kinetics we derive a system of differential equations and compute the steady states using an iterative procedure to eliminate variables. This approach makes it possible to derive exact relationships between concentrations and total amounts at steady state and to study aspects of the system in detail without relying on simulation or numerical evaluations. Our work is an extension of the work in [17], where we gave a detailed mathematical analysis of this cascade at steady state.

The outline of the paper is provided in the following manner. In Sect. 2 we describe the system. In Sect. 3 we give the main mathematical results that we derive about the system. Non-mathematically inclined readers might skip this section. In Sect. 4 we study how species concentrations at steady state vary as a function of the overall substrate and phosphatase levels. We take this further in Sect. 5, where we study stimulus–response and signal amplification. Finally, in Sect. 6, the question of how the maximal response relates to the number of layers as well as the levels of phosphatase or substrate is addressed.

2 One-Site Linear Signaling Cascades

We consider signaling cascades with n layers and a one-site phosphorylation cycle at each layer (Fig. 4.1). The species in each cycle are the *unmodified* substrate S_i^0, the *modified* substrate S_i^1, the *phosphatase* F_i, the *kinase* S_{i-1}^1, and the *intermediate (enzyme–substrate) complexes* Y_i^0 and Y_i^1 for $i = 1, \ldots, n$. That is, in each layer the kinase is the phosphorylated substrate of the previous layer. The kinase of the first

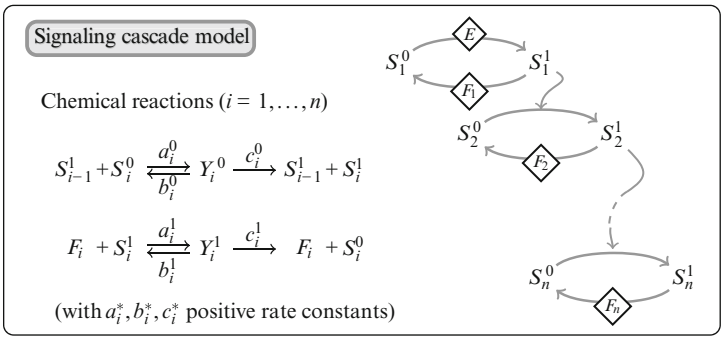


Fig. 4.1 One-site cascade of length n. The enzyme mechanism follows the classical Michaelis–Menten model. In the first layer, the substrate S^1_0 is the kinase E is the first cycle

layer is not a substrate in any other layer and we denote it by $E = S^1_0$ (corresponding to a 0th layer). The modified substrate S^1_i of the ith layer is called the *response* of the ith layer; in particular the response of the nth layer is called the *final* response or simply the response of the cascade.

The system is specified by the set of chemical reactions (Fig. 4.1). The enzyme mechanism follows the classical model of Michaelis and Menten, in which an enzyme–substrate complex is formed reversibly, while its dissociation into product and enzyme is irreversible. Further, the phosphate donor, typically ATP, is assumed in abundance and embedded into the rate constants. This reaction set-up has frequently been used to study signaling cascades, see e.g., [5, 7, 14, 18–20].

2.1 Steady States

Assuming mass-action kinetics, the differential equations describing the dynamical system over time t are given by:

$$\dot{S}^1_i = (b^0_{i+1} + c^0_{i+1})Y^0_{i+1} + c^0_i Y^0_i + b^1_i Y^1_i - (a^0_{i+1}S^0_{i+1} + a^1_i F_i)S^1_i \tag{4.1}$$

$$\dot{S}^0_i = b^0_i Y^0_i + c^1_i Y^1_i - a^0_i S^0_i S^1_{i-1} \tag{4.2}$$

$$\dot{Y}^0_i = -(b^0_i + c^0_i)Y^0_i + a^0_i S^0_i S^1_{i-1} \tag{4.3}$$

$$\dot{E} = (b^0_1 + c^0_1)Y^0_1 - a^0_1 S^0_1 E \tag{4.4}$$

$$\dot{F}_i = (b^1_i + c^1_i)Y^1_i - a^1_i F_i S^1_i \tag{4.5}$$

$$\dot{Y}^1_i = a^1_i F_i S^1_i - (b^1_i + c^1_i)Y^1_i \tag{4.6}$$

for $i = 1\ldots,n$ and where we put $Y^0_{n+1} = S^0_{n+1} = 0$. It follows from Equations (4.5) and (4.6) that $\dot{F}_i + \dot{Y}^1_i = 0$. Similarly, from (4.4) and (4.3) for $i = 1$ we have

that $\dot{E} + \dot{Y}_1^0 = 0$. This implies that the values $F_i + Y_i^1$ and $E + Y_1^0$ are independent of time. Similarly, $S_i^0 + S_i^1 + Y_i^0 + Y_i^1 + Y_{i+1}^0$ is also constant. Hence, the system has the following *conservation laws*:

$$\overline{F}_i = F_i + Y_i^1, \quad \overline{E} = E + Y_1^0, \quad \overline{S}_i = S_i^0 + S_i^1 + Y_i^0 + Y_i^1 + Y_{i+1}^0, \tag{4.7}$$

for $i = 1, \ldots, n$, and $Y_{n+1}^0 = 0$. The quantities $\overline{E}$, $\overline{F}_i$, and $\overline{S}_i$ are called the total amounts of enzymes and substrates, or just the total amounts.

The steady states of the cascade are found by setting the right hand side of (4.1)–(4.6) to zero. The conservation laws imply that the equations corresponding to $\dot{S}_i^0, \dot{E}, \dot{F}_i = 0$ are redundant. Therefore, given total amounts $\overline{E}, \overline{F}_i, \overline{S}_i$, the steady states of the system are the concentrations that fulfill the conservation laws (4.7) (linear equations) together with $\dot{S}_i^1, \dot{Y}_i^0, \dot{Y}_i^1 = 0$ (quadratic equations).

These equations provide a system of polynomial equations with $5n + 1$ equations and variables which, because of the quadratic equations, may have many solutions. However, we are only interested in biologically relevant solutions for which all concentrations at steady state are positive or zero. This suggests the following definition: A *Biologically Meaningful Steady State* (BMSS) is a steady state for which all total amounts are positive and all species concentrations are positive or zero.

In [17], we prove that the cascade has precisely one BMSS for any choice of kinetic rate constants. Further, we show that the BMSS concentrations are in fact positive (i.e., non-zero) and hence each concentration at steady state is strictly smaller than a corresponding total amount, e.g., $E < \overline{E}$. By abuse of language, we often say "the steady state", while meaning the BMSS. Likewise, we say, e.g., "the kinase E fulfills..." when in fact we mean "the concentration of the kinase E fulfills...".

Having set the notation, we can formalize the scope of this work: we seek to study how the BMSS (in particular the response S_i^1) changes when the total amounts $\overline{E}$, $\overline{S}_i$ or $\overline{F}_i$ change, and how a change in one layer effects the responses in other layers.

2.2 Concentrations at Steady State

Using (4.1)–(4.6) together with the conservation laws the following relations apply at steady state,

$$F_i = \frac{\overline{F}_i}{1 + \delta_i S_i^1}, \quad Y_i^1 = \frac{\delta_i \overline{F}_i S_i^1}{1 + \delta_i S_i^1}, \quad Y_i^0 = \frac{\gamma_i \overline{F}_i S_i^1}{1 + \delta_i S_i^1}, \quad S_i^0 = \frac{\lambda_i \overline{F}_i S_i^1}{(1 + \delta_i S_i^1) S_{i-1}^1} \tag{4.8}$$

for $i = 1, \ldots, n$, with constants $\delta_i = a_i^1/(b_i^1 + c_i^1)$, $\gamma_i = (c_i^1/c_i^0)\delta_i$, and $\lambda_i = \gamma_i(b_i^0 + c_i^0)/a_i^0$. The constant δ_i is the inverse of the Michaelis–Menten constant for F_i, γ_i is the catalytic efficiency $c_i^1 \delta_i$ of F_i divided by the dissociation constant c_i^0 of S_{i-1}^1, and λ_i is the relative catalytic efficiency in layer i, that is, the quotient of the catalytic efficiency of F_i by that of S_{i-1}^1.

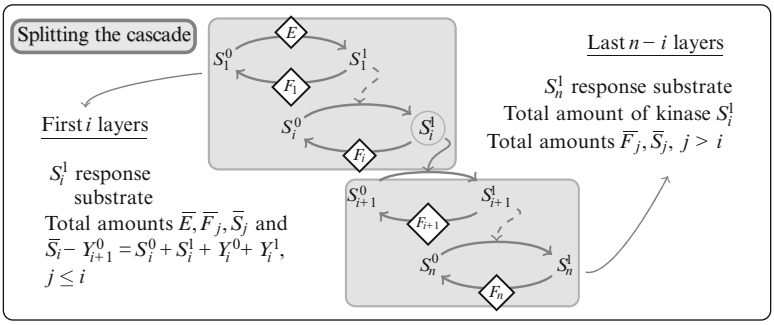


Fig. 4.2 Splitting the cascade at the ith layer

Equation (4.8) is essential: It provides simple relationships between different concentrations at steady state. The values Y_i^0, Y_i^1, and F_i depend only on the rate constants in the ith layer and are increasing in $\overline{F}_i$ and S_i^1. The value S_i^0, however, depends on the steady state values of the modified substrates in the ith and $(i-1)$th layers, providing a link between the two layers.

2.3 Splitting the Cascade

Consider the cascade obtained from the first i layers. Its connection to the last $n-i$ layers is through the intermediate complex Y_{i+1}^0 accounting for the conversion of S_{i+1}^0 to S_{i+1}^1 via the kinase S_i^1. If Y_{i+1}^0 is known, then the steady state concentrations in the first i layers satisfy the steady state equations of a cascade of length i with total amounts $\overline{E}, \overline{F}_1, \ldots, \overline{F}_i, \overline{S}_1, \ldots, \overline{S}_{i-1}$, and $\overline{S}_i - Y_{i+1}^0 = S_i^0 + S_i^1 + Y_i^0 + Y_i^1$. Thus, the intermediate complex Y_{i+1}^0 influences the layers upstream of layer $i+1$ by reducing the total amount of substrate available at layer i. This effect is known as sequestration.

Similarly for the cascade consisting of the last $n-i$ layers. If S_i^1 is known (fixed), then the steady state concentrations in the layers $i+1, \ldots, n$ satisfy the steady state equations of a cascade of length $n-i$ with total amounts $\overline{F}_{i+1}, \ldots, \overline{F}_n$, $\overline{S}_{i+1}, \ldots, \overline{S}_n$ and total amount of kinase S_i^1.

This split is illustrated in Fig. 4.2. The results presented in the following sections rely on splitting the cascade in this way.

3 Relationships Between Response Concentrations

In this section we provide an iterative expression for the ith response S_i^1 in terms of the final response S_n^1. Some consequences of this result are discussed in the forthcoming sections.

3.1 The Last Layer

If the expressions in (4.8) are substituted for Y_n^0, Y_n^1, S_n^0 in the conservation law $\overline{S}_n = S_n^0 + S_n^1 + Y_n^0 + Y_n^1$ we obtain S_{n-1}^1 as an (increasing) function of S_n^1,

$$S_{n-1}^1 = f_{n-1}(S_n^1) = \frac{\lambda_n \overline{F}_n S_n^1}{d_n(S_n^1, 0)},$$

with $d_i(x, y) = (\overline{S}_i - y) - x - \overline{F}_i(\delta_i + \gamma_i)x + \delta_i(\overline{S}_i - y)x - \delta_i x^2$, $1 \leq i \leq n$. If S_n^1 is positive, then S_{n-1}^1 is positive provided $d_n(S_n^1, 0)$ is positive. This is the case only if $S_n^1 \in [0, \alpha_n)$, where α_n is the only positive root of $d_n(x, 0)$. Therefore, $\overline{F}_n$, $\overline{S}_n$, and the rate constants of layer n restrict S_n^1 at steady state, $S_n^1 < \alpha_n$, independently of the parameters in the other layers.

If S_n^1 is close to α_n, the denominator of f_{n-1} is close to zero and hence S_{n-1}^1 is large. Since the amount of substrate in layer $n-1$ is bounded by $\overline{S}_{n-1}$, S_{n-1}^1 cannot be arbitrarily large. Thus, upstream layers limit the possible values of S_n^1 further.

3.2 Intermediate Layers Response

In (4.8), S_{i+1}^1 gives Y_{i+1}^0. This observation allows us iteratively to calculate all responses S_i^1 as functions of S_n^1. Specifically, consider the ith layer of the cascade. For every $Y_{i+1}^0 < \overline{S}_i$, the steady state values of the species in the first i layers are found by solving the steady state equations for the cascade consisting of the layers from 1 to i with the total amount of substrate in layer i being $\overline{S}_i - Y_{i+1}^0$. Therefore, we obtain

$$S_{i-1}^1 = g_i(S_i^1, Y_{i+1}^0) = \frac{\lambda_i \overline{F}_i S_i^1}{d_i(S_i^1, Y_{i+1}^0)}, \tag{4.9}$$

with $g_n(S_n^1, Y_{n+1}^0) = f_{n-1}(S_n^1)$, since $Y_{n+1}^0 = 0$. The response S_{i-1}^1 can be found in terms of S_n^1 by repeated application of (4.9). Positivity of S_{i-1}^1 imposes an upper bound β_{i-1} to S_n^1, which is smaller than the upper bound β_i imposed by S_i^1. Indeed, when S_n^1 is close to β_i, S_i^1 is large and then d_i becomes negative.

We have outlined the following result, which is proven in [17, Prop. 2.31].

Result 1 (Response relationships) *For $i = 0, \ldots, n-1$, the BMSS value of S_i^1 satisfies $S_i^1 = f_i(S_n^1)$, where f_i is an increasing function of S_n^1 defined on an interval $[0, \beta_i)$. Furthermore,*

- *$\beta_i < \beta_{i+1}$ and $\beta_i < \beta_{n-1} = \alpha_n$ for $i < n-1$. β_i depends on $\overline{F}_j, \overline{S}_j$, $j \geq i+1$ only.*
- *Let α_i be the positive root of $d_i(x, 0)$ which depends on $\overline{F}_i$ and $\overline{S}_i$. Then $S_i^1 < \alpha_i$ for any BMSS.*

This result is important and shows how each additional layer further constrains the maximal value of S_n^1. Also, the response S_i^1 is bounded by α_i, which depends exclusively on the rate constants and total amounts of layer i. This upper bound is obtained by ignoring sequestration, i.e., assuming $Y_{i+1}^0 = 0$.

Result 1 provides an iterative procedure for calculating response relationships. All terms that appear in the function f_i are mathematically simple (polynomials) and hence f_i is a rational function. Such functions are easy to manipulate, for example, using programs like Mathematica™.

3.3 Total Amount of Kinase $\overline{E}$

Using Result 1 we obtain the increasing relations $E = S_0^1 = f_0(S_n^1)$ and $Y_1^0 = \frac{\gamma_1 \overline{E}_1 f_1(S_n^1)}{1+\delta_1 f_1(S_n^1)}$. The latter we denote $Y_1^0 = f_1^Y(S_n^1)$. These functions do not dependent on the stimulus $\overline{E}$ and hence

$$\overline{E} = r\left(S_n^1\right) = f_0\left(S_n^1\right) + f_1^Y\left(S_n^1\right)$$

gives $\overline{E}$ as an increasing function of S_n^1. The function f_0 is defined for $S_n^1 < \beta_0$ and tends to infinity as S_n^1 tends to β_0. The function f_1^Y is defined for $S_n^1 < \beta_1$. Since $\beta_0 < \beta_1$, the function r is increasing and defined for $S_n^1 \in [0, \beta_0)$. It tends to infinity when S_n^1 tends to β_0.

As a consequence, for positive $\overline{E}$, there is a unique value of S_n^1 satisfying the relation $\overline{E} = r(S_n^1)$. This is the BMSS value of S_n^1. All other concentrations can be derived from this using (4.8) and the functions g_i. Further, the upper bound β_0 of S_n^1 is only obtained if $\overline{E}$ is very large. We introduce a distinctive symbol for this upper bound, or the *maximal response* of the cascade: $\sigma_n := \beta_0$. Writing r as a quotient of polynomials, σ_n is simply the first positive root of the denominator.

4 Regulation Through Substrate and Phosphatase Variation

In the previous section we found S_i^1 in terms of S_n^1. This relation provides means to explore how noise and regulation at intermediate layers (e.g., *crosstalk* [14]) propagate upstream and downstream in the cascade and effects the responses.

The following result is from [17, Th. 2.32, Th. 2.33] and illustrated in Fig. 4.3a.

Result 2 (Variation in the total amount of substrate) *Consider a cascade with n layers and fix all total amounts but $\overline{S}_i$ for some layer i. Then an increase of $\overline{S}_i$ causes*:

- *The BMSS values of the response S_j^1 and the intermediate complexes Y_j^0 and Y_j^1 increase downstream of layer i, that is, for layers $j = i, \ldots, n$.*

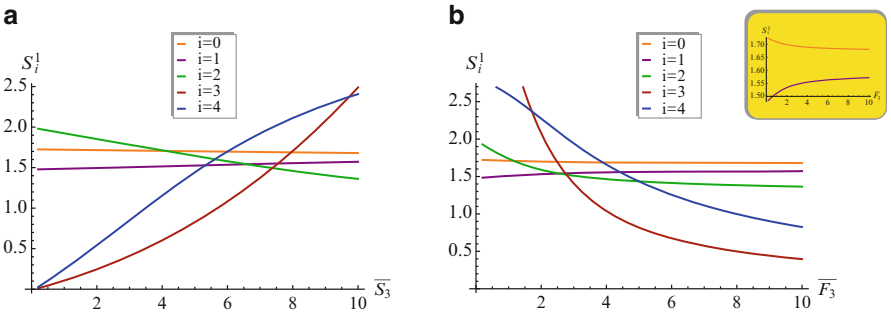


Fig. 4.3 Variation of S_i^1 when the total amounts of phosphatase or substrate are varied. Fixed parameters: $a_*^* = b_*^* = c_*^* = 1, \overline{F}_* = 3, \overline{E} = 3, \overline{S}_* = 7$

- *The BMSS value of the response S_j^1 increases upstream of layer i if $j = 1, \ldots, i-1$ has the same parity as i and decreases otherwise.*

Thus an increase in the total amount of substrate in one intermediate layer propagates downstream as an increase in the concentrations of the modified substrates. This corresponds to increasing the initial kinase or stimulus S_i^1 in the smaller cascade consisting of the layers below the one undergoing variation. However, these layers have fixed total amounts and the modified substrates downstream are therefore bounded by their respective α_i (Result 1).

Also, an increase in the total amount of substrate in an intermediate layer propagates upstream in an alternating fashion. If S_i^1 is increased, so is the sequestered substrate Y_i^0 and hence the total amount at layer $i-1$, $\overline{S}_{i-1} - Y_i^0$ decreases. In turn, this causes S_{i-1}^1 to decrease. In turn, this causes Y_{i-1}^0 to decrease and hence $\overline{S}_{i-2} - Y_{i-1}^0$ to increase and so S_{i-2}^1 increases. This effect is strongly dependent on the intermediate complexes and cannot be demonstrated in a model without these.

Similarly, Result 1 provides insight into how the response varies when the total amount of phosphatase is changed (see Appendix A for a proof).

Result 3 (Variation in total amount of phosphatase) *Consider a cascade with n layers and fix all total amounts but $\overline{F}_i$ for some layer i. If the total amount of phosphatase at layer i, $\overline{F}_i$, is increased then*:

- *The BMSS value of the response S_j^1 decreases downstream of layer i, that is, for $j = i, \ldots, n$.*
- *The BMSS value of the response S_j^1 increases upstream of layer i if $j = 1, \ldots, i-1$ has the same parity as i and decreases otherwise.*

Result 3 is illustrated in Fig. 4.3b. As expected, an increase of phosphatase at layer i causes the amount of phosphorylated substrate at layer i to decrease and likewise all downstream responses to decrease too. In particular, the final

response decreases. Thus, controlling the level of phosphatase at any layer serves as a regulator of the response level. Upstream of layer $i-1$ the response increase/decrease in an alternating way, using the same argument as above.

5 Stimulus–Response Curves

The relationship between stimulus and response has been studied extensively, e.g., [7,11,14,16,21]. Much attention has been devoted to whether a system exhibits ultrasensitivity, that is, whether it reacts to input in a switch-like mode [18,22].

The plot of S_n^1 (the final response) against $\overline{E}$ (the stimulus) is usually called the *stimulus–response* curve. We showed in Sect. 3 that the stimulus and the response are related by an increasing function

$$\overline{E} = r(S_n^1)$$

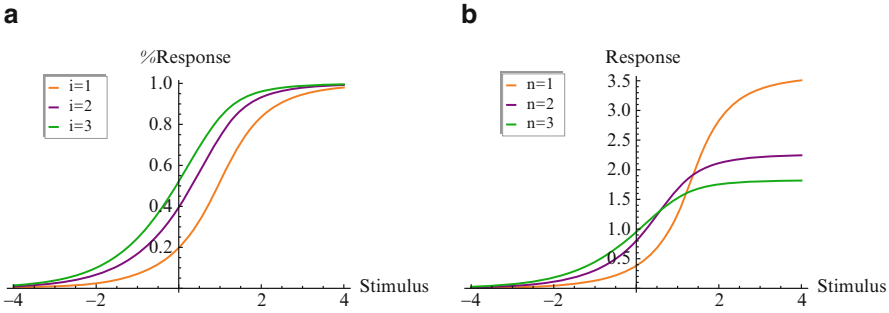
defined on an interval $[0, \sigma_n)$. Thus, the inverse of r is the stimulus–response curve.

When the stimulus $\overline{E}$ is arbitrarily large the final response S_n^1 saturates at its maximal value σ_n. The stimulus required to achieve a certain percentage of the maximal response can be determined from the explicit expression of the inverse stimulus–response curve. Let $\overline{E}_M$ be the value of $\overline{E}$ required to obtain $M\%$ of the maximal response, that is, $\overline{E}_M = r(M\sigma_n/100)$. For instance, 90% of the maximal response is obtained with $\overline{E}_{90} = r(0.9\sigma_n)$. This provides means to compute measures of sensitivity and switch behavior of biological systems: the *response coefficient* (also called cooperativity index) $R = \overline{E}_{90}/\overline{E}_{10}$ [5], the *switch value* $\overline{E}_{90} - \overline{E}_{10}$ [18], and the *Hill coefficient* $n_H = \log(81)/\log(\overline{E}_{90}/\overline{E}_{10})$ [23].

The maximal response σ_i of S_i^1 is easily derived from the maximal response σ_n using $\sigma_i = f_i(\sigma_n)$. Now consider the response in any layer normalized with its maximal response, that is, the normalized, or relative, response is between 0 and 1. We provide conditions for which the normalized response increases when moving down the layers in a cascade for a fixed stimulus $\overline{E}$, Fig. 4.4a. In other words, the normalized stimulus–response curves are shifted to the left as we move down the layers. This is known as signal amplification.

Result 4 (Signal amplification) *If* $1 > \delta_i \overline{S}_i - (\delta_i + \gamma_i)\overline{F}_i$, *then the level of kinase* $\overline{E}$ *required to achieve* $M\%$ *of the maximal response at layer* i *is always smaller than the amount of kinase required to achieve* $M\%$ *of maximal response at layer* $i-1$.

Thus, if the level of phosphatase is in excess relatively to the substrate in all layers, then for any given amount of stimulus, the last layer will always have a higher relative response than the intermediate responses, and the response in the first layer will always have the lowest relative response.



Shift of the normalized stimulus-response curves for the modified substrates S_{i}^1, i = 1, 2, 3, for $n = 3$.

Reduction of the maximal value of response for different cascade lengths $n = 1, 2, 3$ with parameters in the last layer fixed.

Fig. 4.4 Stimulus–response curves in semi-log scale, $\log(\overline{E})$ versus S_n^1. Fixed parameters: $a_*^* = b_*^* = c_*^* = 1, \overline{F}_1 = 5, \overline{F}_2 = 4, \overline{F}_3 = 5; \overline{S}_1 = 8, \overline{S}_2 = 9, \overline{S}_3 = 10$

6 Maximal Response

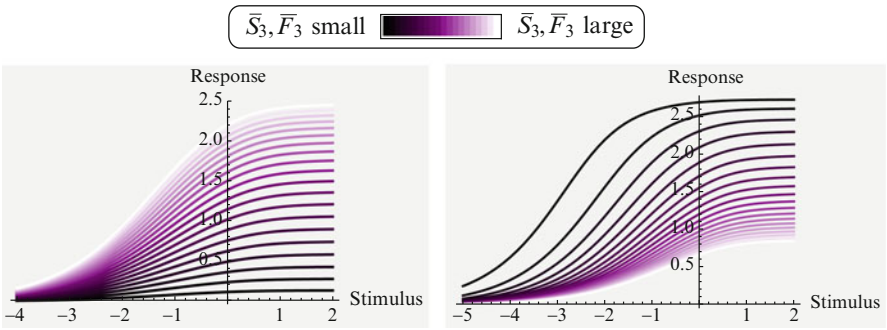
The maximal response is restricted by the total amount $\overline{S}_n$ in the last layer and further by any additional layer, as described in Result 1. The reduction of the maximal response is exemplified in Fig. 4.4b for three cascades with one, two, and three layers. The maximal response of the single-layer cascade is 3.58, but after adding one (respectively two) additional layer(s) on top of it, the maximal response drops to 2.23 (respectively 1.83), which is much lower than the upper bound set by the total amount (fixed to 10). The decline of the maximal response is caused by substrate sequestration: In layers above the last layer, substrates are trapped in intermediate complexes and therefore not able to participate as kinases driving the cascade of modifications that ultimately results in phosphorylation of S_n^0.

How the maximal response changes with changing total amounts of phosphatase and substrate can be quantified. It is stated below and illustrated in Fig. 4.5 (a proof can be found in Appendix A).

Result 5 (Maximal response) *Consider a cascade of length n.*

- *If the total amount of phosphatase at layer i, $\overline{F}_i$, increases then the maximal response σ_n decreases.*
- *If the total amount of substrate at layer i, $\overline{S}_i$, increases then the maximal response σ_n increases.*

Interestingly, an increase in the level of phosphatase at any layer cannot be compensated fully by an increase in the stimulus. Only locally, for low responses, such a loss could be overcome.



Increase of $\overline{S}_3$ causes the maximal response to increase.

Increase of $\overline{F}_3$ causes the maximal response to decrease.

Fig. 4.5 Variation of the maximal response when $\overline{S}_3$ and $\overline{F}_3$ are increased. $\overline{S}_3, \overline{F}_3$ are increased from 0.5 to 10. Fixed parameters: $a_*^* = b_*^* = c_*^* = 1; \overline{F}_* = 3; \overline{E} = 3; \overline{S}_* = 7$

7 Discussion

We have provided a theoretical discussion of linear cascades with arbitrary number of layers of one-site phosphorylation cycles. In particular, we have focused on *intrinsic* properties of the cascade, that is, properties that do not rely on specific reaction rate constants. Such studies may be useful for testing new hypotheses, since experimental data is difficult to obtain and rate constants are hard to estimate [24, 25].

Many cascades are regulated externally, but the effect of such regulation is generally unclear. Our study sheds light on how the steady state changes as a consequence of changing levels of phosphatases and substrates. If a level increases at some layer in the cascade, then all responses downstream decrease (phosphatase) or increase (substrate). Thus, regulation at each layer of the final response is possible. Further, the maximal response (obtained when stimulus is very large) follows the same pattern. An increase in the phosphatase level at some layer causes the maximal response to decrease. This loss cannot be compensated for by an increase in the stimulus.

Upstream of the modified layer variations in the responses follow an alternating behavior: If the response at some layer above the modified layer increases, the response in the next layer decreases, and so forth. This behavior is counterintuitive: The response of one layer is the kinase of the next layer, and we might expect the same qualitative change in each layer. However, the result relies strongly on the formation of intermediate complexes and thus relates to (hidden) sequestration. It is therefore important, that intermediate complexes are modeled explicitly as in our approach.

Under some conditions, signal amplification also occurs in the cascade in the sense that the relative response increases down through the cascade. Thus, in a long

cascade the final response can come up faster than in a short cascade. However, this gain in signal amplification has to be contrasted to a reduction of the maximal response with increasing cascade length. Consequently, the cascade length is a compromise between when and how high the final response should be.

A deeper study is required to understand to what extend this balance between gain and lost is beneficial for the cell. It may depend on the specific levels of phosphatase and substrate as well as on the reaction rate constants. Although the results presented here are qualitatively independent of the rate constants, their effect is crucial in determining the magnitude of a change.

Acknowledgements EF has received support from a postdoctoral grant of the "Ministerio de Educación" of Spain and the project MTM2009-14163-C02-01 from the "Ministerio de Ciencia e Innovación". CW is supported by the Lundbeck Foundation, Denmark.

Appendix

Proofs

The proofs follow very closely the proofs for Results 1 and 2 which can be found in our previous paper [17].

Proof of Result 3. In the sequel, we assume that all total amounts but $\overline{F}_i$ are fixed. Consider a cascade of length n and fix a value of S_n^1. Define

$$Y_i^0 = g_i^Y(S_i^1) = \frac{\gamma_i \overline{F}_i S_i^1}{1 + \delta_i S_i^1}. \tag{4.10}$$

Using (4.10) and (4.9), we see that for $j \geq i$, S_j^1, Y_{j+1}^0 are independent of $\overline{F}_i$. Then, by (4.10), Y_i^0 is an increasing function of $\overline{F}_i$, and so is S_{i-1}^1 by (4.9) (there might be singularities). For $j \geq i-2$, S_j^1, Y_j^0 are increasing in S_{j+1}^1, Y_{j+2}^0 (with expressions not involving $\overline{F}_i$). We conclude that they are increasing in $\overline{F}_i$ for fixed S_n^1. It follows that the steady state value of S_n^1 must decrease if $\overline{F}_i$ is increased. Indeed, we have $\overline{E} = E + Y_1^0$ with E, Y_1^0 increasing both in S_n^1 and $\overline{F}_i$. Since the functions $f_i, \ldots, f_{n-1}$ are independent of $\overline{F}_i$ and increasing in S_n^1, the concentrations S_j^1 for $j = i, \ldots, n$ decrease in $\overline{F}_i$.

As shown in [17], the BMSS of a cascade of length n satisfies $S_n^1 = \psi(\overline{S}_n)$, with ψ an increasing continuous function defined over the non-negative real numbers. Hence, if we consider now the split of the cascade at layer $i-1$, the steady state value of S_{i-1}^1 is given by a decreasing continuous function of Y_i^0, $S_{i-1}^1 = f(Y_i^0) := \psi(\overline{S}_{i-1} - Y_i^0)$, obtained by considering the first $i-1$ layers of the cascade with total amounts $\overline{E}, \overline{F}_1, \ldots, \overline{F}_{i-1}, \overline{S}_1, \ldots, \overline{S}_{i-2}$, and $\overline{S}_{i-1} - Y_i^0$. The function f is independent of $\overline{F}_i$.

Let now $h(\overline{F}_i)$ denote the value of Y^0_{i+1} at steady state, corresponding to the total amount $\overline{F}_i$. By (4.9), and writing S^1_i as a function of Y^0_i using (4.10), we have that $S^1_{i-1} = \widetilde{g}_i(Y^0_i, Y^0_{i+1}) = \widetilde{g}_i(Y^0_i, h(\overline{F}_i))$. If we write

$$\widetilde{g}_i(Y^0_i, Y^0_{i+1}) = \frac{p_1(Y^0_i, Y^0_{i+1})}{p_2(Y^0_i, Y^0_{i+1})},$$

then $p_1(y,z) = \lambda_i y(\xi - y)$, and $p_2(y,z) = (\delta_i + \gamma_i)y^2 - \gamma_i(1/\delta_i + \overline{F}_i + \xi + (\overline{S}_i - z))y + \gamma_i\xi(\overline{S}_i - z)$ with $\xi = \gamma_i\overline{F}_i/\delta_i$. Computing the partial derivative of this function with respect to Y^0_{i+1} and $\overline{F}_i$, we see that $\widetilde{g}_i$ is decreasing in $\overline{F}_i$ and increasing in Y^0_{i+1}. Since h is decreasing in $\overline{F}_i$, it follows that $\widetilde{g}_i(Y^0_i, h(\overline{F}_i))$ is decreasing in $\overline{F}_i$ for any fixed Y^0_i.

The steady state value of the pair (Y^0_i, S^1_{i-1}) for a fixed $\overline{F}_i$, must satisfy both equalities $S^1_{i-1} = f(Y^0_i) = \widetilde{g}_i(Y^0_i, h(\overline{F}_i))$. Since f is independent of $\overline{F}_i$ and $\widetilde{g}_i$ decreases in $\overline{F}_i$, we have that Y^0_i increases in $\overline{F}_i$ while S^1_{i-1} decreases.

It follows that Y^0_{i-1} decreases too. If for $j \le i-1$, Y^0_j increases, then the total amount of layer $j-1$, $\overline{S}_{j-1} - Y^0_j$ decreases and thus S^1_{j-1} decreases. On the contrary, if Y^0_j decreases, then the same arguments shows that S^1_{j-1} increases completing the proof. □

Proof of Result 5. Let $\sigma_n(\overline{F}_i)$ denote the maximal response of S^1_n corresponding to the total amount of phosphatase $\overline{F}_i$. Similarly, denote by $\beta_j(\overline{F}_i)$ the upper bounds of Result 1. Note that d_j is decreasing in $\overline{F}_j$. Since S^1_n decreases in $\overline{F}_i$, $\sigma_n(\overline{F}_{i,1}) \le \sigma_n(\overline{F}_{i,2})$ if $\overline{F}_{i,1} > \overline{F}_{i,2}$. The question is whether they can be equal or not.

Fix $S^1_n = \sigma_n := \sigma_n(\overline{F}_{i,2})$. Let $\overline{\rho}_1(S^1_n) = \rho_1 \circ f^Y_2(S^1_n)$ be defined as the positive root of the polynomial $d_1(x, f^Y_2(S^1_n))$. The maximal response $\sigma_n = \beta_0$ is given by the positive value of S^1_n for which $f_1(S^1_n) = \overline{\rho}_1(S^1_n)$. Thus, we have

$$f_1(\sigma_n, \overline{F}_{i,2}) = \overline{\rho}_1(\sigma_n, \overline{F}_{i,2}), \tag{4.11}$$

where we add the reference to the total amount of phosphatase. As noted in the preceding proof, if σ_n is fixed and $\overline{F}_i$ is increased, then f_1 is an increasing function. Similarly, $f^Y_2(\sigma_n, \overline{F}_i)$ is increasing too, and since $\rho_1(Y^0_2)$ is decreasing in Y^0_2, the function $\overline{\rho}_1$ is decreasing in $\overline{F}_i$. Note that since d_1 decreases in $\overline{F}_1$, the argument applies even if $i = 1$.

It follows that if $\overline{F}_{i,2}$ satisfies equality (4.11), then the equality cannot be satisfied by $\overline{F}_{i,1} \neq \overline{F}_{i,2}$ and the first part of the result follows.

The same reasoning applies to the maximal response following variation on the total amount $\overline{S}_i$ at some layer i. By Result 2, S^1_n increases if $\overline{S}_i$ increases, and thus, using the corresponding notation, we have $\sigma_n(\overline{S}_{i,1}) \le \sigma_n(\overline{S}_{i,2})$ if $\overline{S}_{i,1} < \overline{S}_{i,2}$. It is easy to see that we can proceed as above to rule out equality. One just have to observe that if $\overline{S}_i$ increases, then, for fixed $S^1_n = \sigma_n$, both $f_1, \overline{\rho}^1$ are decreasing functions. □

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